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Screening for the Presence of *Salmonella* Typhimurium in Local Meat using Dot-ELISA

Noor Azlina Masdor*[1], Azizah Abdul Talib[2], Noorshinah Hussin[1], Aseha Yahaya[1] and Zamri Ishak[1]

[1]Biotechnology Research Centre, MARDI, 43400 Serdang, Selangor
[2]Inno Biologics Sdn Bhd, Lot 1, Persiaran Negeri BBN, Putra Nilai, 71800 Nilai, Negeri Sembilan

*Corresponding author: Noor Azlina Masdor; Tel No: 03-8943 5942; 03-8945 6037; email: azlina@mardi.gov.my/ azlina.mardi@gmail.com

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Abstract

Salmonella infections in commercial poultry have long been an industry concern and the subject of many investigations. Since poultry is a major food source, its contamination with salmonellae may result in the development of human illness. *Salmonella* Typhimurium is one of paratyphoid *Salmonellae* most commonly associated with poultry. Thus, a detection assay for this bacterium is highly sought after. Detection of *Salmonella* Typhimurium using monoclonal antibody is specific to only one epitope while polyclonal antibody has the ability to detect various serovars due to the presence of multitude of epitopes. In this study the production of polyclonal antibody was performed using rabbits immunized with formalin-killed cell lysate of *Salmonella enterica* subsp. *enterica serovar* Typhimurium ATTC® 53648. The purification of immunoglobulin G (IgG) was carried out by affinity chromatography and the purity of IgG was characterized by SDS-PAGE. The purified IgG was used to detect *Salmonella* Typhimurium by the dot-ELISA method. The specificity of the dot-ELISA was investigated with different foodborne pathogens including *Escherichia coli* O157:H7 and *Campylobactor jejuni* which produced no significant reaction signal compared to *Salmonella* Typhimurium.

INTRODUCTION

Salmonella is recognized as an important foodborne pathogen worldwide. It can be isolated from raw meat, poultry, poultry products, milk and milk products [1]. *Salmonella* outbreaks have been associated with poor cooking, reheating of foods and improper handling of foods. Diseases caused by *Salmonella* are generally called salmonellosis and include typhoid fever- which is the most lethal [2]. *Salmonella* Typhimurium is one of salmonellae paratyphoid most commonly associated with poultry. Thus, a detection assay for this bacterium is highly sought after. Conventional procedure for the detection of *Salmonella* is by the culture method. However, cultural isolation and identification requires three to five days for completion and includes tedious pre-enrichment, selective enrichment, selective agar plating, biochemical screening and serological identification [3]. Therefore, an immunological method has become widely used it is rapid, specific, highly sensitive and can detect a small amount of *Salmonella* in a sample [4]. Some methods may take only 2-3 hours for analysis, such as the ELISA, immuno-magnetic separation and the immuno-precipitation methods. However, the dot-ELISA method is a method of choice because it is easy to use, economical and highly sensitive [5]. In addition, it does not require special equipment and uses lower amounts of reagents. In this study, dot blot test strip kit based on ELISA has been developed for the detection of *Salmonella* Typhimurium in poultry products. The advantage of this kit is that the results obtained are easily interpreted without an ELISA reader, simple, rapid and inexpensive.

MATERIALS AND METHODS

Reference strains and preparation of antigen

Salmonella enterica subsp. *Enterica serovar* Typhimurium ATTC® 53648 was obtained from American Type Culture Collection (ATCC). The preparation of immunogen for the production of a polyclonal antibody against *Salmonella* Typhimurium was performed as described by Siragusa and Johnson [4] with some modifications. For immunogen set up, *Salmonella* Typhimurium was cultured in NB at 37 °C for 16 hours with shaking and $1x10^9$ cells obtained were treated (inactivated) with 0.5% formalin for 24 hours followed by centrifugation (3,000 x g, 4 °C) for 30 min. The precipitate was washed 3 times with 0.01 M phosphate buffered saline (PBS) and finally suspended in 1 ml PBS. The immunogen was kept at -20 °C and used in further experiment.

Immunization procedures

New Zealand white rabbits were injected sub-cutaneously with an emulsion consisting of 0.5 mg of the immunogen dissolved in 0.5

ml of PBS and an equal volume of Freud's complete adjuvant. The injections were repeated three times weekly after the initial injection, substituting Freund's incomplete adjuvant for complete adjuvant. A booster injection was given one month after the initial injection and was repeated at monthly intervals thereafter. The rabbits were bled for antibody titer determinations 2 weeks after each boost.

Antibody purification

Antisera was separated from red blood cells (RBC) by centrifugation of the blood in serum clot activator tube (Vacuette). Antisera at the upper phase of the tube was collected and diluted with water (1:10) and were then precipitated with saturated ammonium sulphate, centrifuged, dialyzed and eluted through a protein a sepharose affinity chromatography column and monitored using an akta purifier instrument (Pharmacia Ltd). Fractions giving the highest absorbance reading at OD_{280nm} were collected and freeze dried (IgG stock). IgG titers for anti-*Salmonella* Typhimurium antibody was determined by indirect ELISA method. Further purity of IgG was checked using SDS-PAGE.

Indirect ELISA method for detemination of antibody titer

Microtiter plates were coated by adding 100 µL of coating heat killed *Salmonella* Typhimurium cell at 10^8 dilution in 0.1 M carbonate buffer, pH 9.6 mixed with 1:20 milk as a blocker and incubate overnight at 4 ^oC. The plate was emptied and washed three times with 200 µL of PBS-Tween. Unoccupied sites on the polystyrene well surface were blocked by treating with 1:10 (v/v) solution of milk diluent in 100 µL of PBS for 30 min at 37 ^oC. Then, the plate was emptied and washed three times with 200 µL of PBS-Tween. Purified *Salmonella* Typhimurium antibodies ranging from 1: 4000, 1:8000, 1:64000 and 1: 128000 were added and the plate was incubated 2 hours at 37 ^oC. The plate was emptied and washed three times with PBS-Tween. Secondary antibody solution anti-rabbit conjugated with hrp was diluted in pbs (1 μgmL^{-1}) and was added (100 µL per well) to the plate. The plate was incubated for 30 minutes at 37 ^oC. The plate was emptied and washed three times with 200 µL of PBS-Tween. TMB Substrate (tetramethylbenzidine substrate solution, KPL products) was added to the plate (100 µL per well). The reaction was stopped after 30 minutes by adding 50 µl of 1 m H_2SO_4 and the absorbance at 405 nm was measured using Dynex revelation 4.21 Microplate Reader. The intensity of the color was proportional to the amount of antibody present.

Dot-ELISA format

Discs (5 mm) were punched from sheets of nitrocellulose membranes with a perforator. The discs were placed in petri dish and 5 µL of antigen was spotted onto the discs. The antigen was prepared from each individual culture fluids of bacteria filtered through 0.45 µm filter disc. The NC membrane was allowed to dry at room temperature followed by the addition of blocking agent consisting of 1% skim milk for 1 hour. Excess skim milk was washed away using PBST and the *Salmonella* Typhimurium antibodies (0.000001 to 0.5 mg/ml) were then added onto the membrane. Excess antibody was washed away with PBST. Then the membrane was incubated for 30 minutes with a secondary antibody (alkaline phosphatase-conjugated goat anti-rabbit IGg). The membrane was again washed with excess PBST and incubated with Bcip. Discs with positive reactions showed a distinct blue spot.

Conventional detection method

For the selective enrichment method, about 1.0 mL and 0.1 mL of cultures grown in LB (Difco) were transferred to tetrathionate (TT) (Difco) broths and Rappaport Vassiliadis (RV) (Difco Laboratories, Detroit, MI), respectively. These cultures were incubated at between 42 ºC (RV) and 43ºC (TT) for between 18 and 24 h. Then the broths were plated in bismuth sulphite agar (Difco) and enteric agar (Difco) and incubated at 37 ºC for 18 - 24h. Biochemical identification by cultivation at 37 ºC for 24 h in Triple Sugar Iron agar (TSI) (Difco), lisine iron agar (LIA) (Difco) and urea broth (Difco) was then carried out.

RESULTS

Measurement of antibody titer

The results shows that the antibody production was successful compared to pre-immune (Figure 1). This suggests that the immunization and purification method used were successful. The highest antibody production was at the post booster II reaching an absorbance of 1.98 at dilution 1:16000 while higher serum dilutions (>1:1024000) resulted in a weaker response.

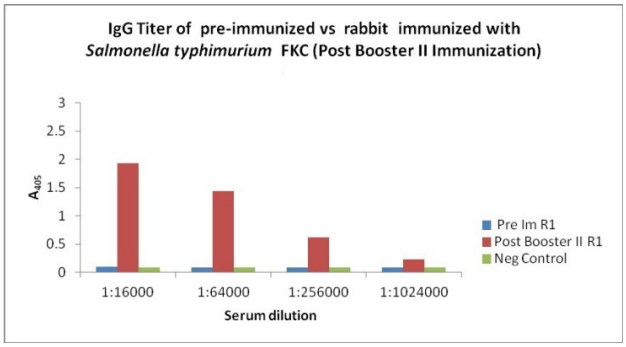

Figure 1: Comparison of IgG titer between pre-immunized and booster II serum

Purification of IgG

The purity of IgG was determined by SDS-PAGE, which showed two bands of 50 kDa and approximately 25 kDa which were heavy chain and light chain of IgG, respectively (Figure 2).

Determination of specificity and optimization of concentration of purified IgG by the dot-ELISA method

The dot-ELISA method was carried out as described using the purified IgG to test the responses to *Salmonella* Typhimurium. The optimum concentration of IgG was at 0.0001 mg/ml showing a clear blue color on the nitrocellulose membrane (Table 1).

Determination of specificity to various serovars of *Salmonella* and determination of cross reaction to other enteric Gram-negative bacteria by the dot-ELISA method

The dot-ELISA method was used to determine the specificity to various serovars of *Salmonella* and tested for cross reaction with other enteric Gram-negative bacteria (*E. coli* O157:H7 and *Campylobacter jejuni*). The results were positive for all *Salmonella* tested and negative for other enteric Gram-negative bacteria (Table 2).

Locally obtained meat samples were then tested for both methods (Table 3).

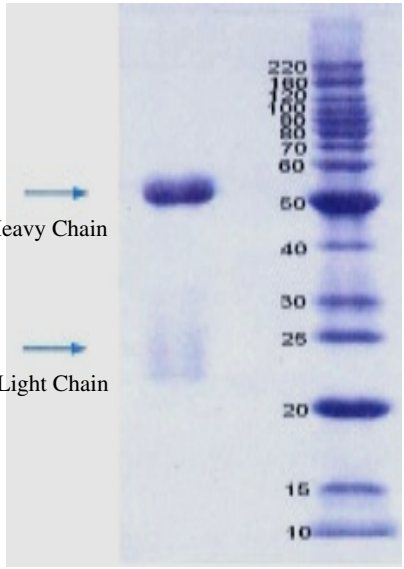

Figure 2: Reducing SDS-PAGE of the anti-*Salmonella* Typhimurium stained with Coomassie Blue (14% gel, 1500 µg protein per lane)

Table 1: Concentrations of immunoglobulin G (IgG) producing different blue intensities on nitrocellulose membrane

Concentration of purified IgG (mg/mL)	Dot-ELISA results
0.5	
0.1	
0.01	
0.001	
0.001	
0.00001	
0.000001	

Table 2. Detection of various serovars of *Salmonella* and other enteric Gram-negative bacteria by the dot-ELISA method (+ indicates detected)

Microorganism	Result
Salmonella gallinarum	+
Salmonella pullorum	+
Salmonella enteritidis	+
E. coli O1H7	-
Campylobacter jejuni	-

Table 3: Detection of *Salmonella* in 15 chicken meat sample by dot-blot test strip kit and conventional method.

Samples	*Salmonella* dot-blot test strip kit	Conventional method
Meat 1	Detected	Detected
Meat 2	Detected	Detected
Meat 3	Detected	Not detected
Meat 4	Detected	Detected
Meat 5	Detected	Not detected
Meat 6	Detected	Detected
Meat 7	Detected	Detected
Meat 8	Detected	Not detected
Meat 9	Detected	Detected
Meat 10	Detected	Detected
Meat 11	Detected	Detected
Meat 12	Detected	Detected
Control positive	Detected	Detected
Control negative	Not detected	Not detected

DISCUSSION

In the present study, various conditions were optimized for the detection of *Salmonella* Typhimurium through dot-ELISA using rabbit anti-*Salmonella* Typhimurium IgG. The optimum and economic concentration of antibody was at 0.0001 mg/ml showing a clear blue color on the nitrocellulose membrane. A previous publication had reported a much lower sensitivity with the lowest detected observable blue dot at 0.005 mg/ml [6]. The results of the specificity test on the various serovars of *Salmonella* shows that the antibody produced could detect *Salmonella pullorum, Salmonella gallinarum* and *Salmonella enteritidis* suggesting the ability of the produced antibody to detect a relatively wide spectrum of *Salmonella* species. Compared to monoclonal antibodies, polyclonal antibodies could detect numerous epitopes on the cells of bacteria especially from the same genus. Although this is an advantage in terms of multi species detection, cross reaction to other genus within the same bacterial family does occur with high frequency [6]. However, test on cross reactions showed no reaction occurred with other enteric Gram-negative bacteria *(E. coli O157:H7* and *Campylobacter jejuni*). The fact that cross reaction was not observed in this work indicates that we had developed a sensitive and interference free detection kit.

CONCLUSION

The developed dot-ELISA method for the detection of *Salmonella* showed promising results with negative interference from other foodborne pathogens indicating specificity. The method is suitable for rapid detection of *Salmonella* in food samples, water and other drinks**.**

Acknowledgement

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